REMARKS

Applicants have further amended the claims in order to more clearly define the invention taking into consideration the outstanding Official Action. Claims 26 and 42 have been amended to make it clear that it is the cereal that has the enzymatic activity to produce or induce the defined level of antisecretory proteins (ASP) in the blood. Moreover, the claims have been amended to make it clear that a mixture of cereals is not required to obtain the results as fully supported by Applicants' specification, see for example page 6 of applicants' specification where malted oat flakes were consumed. New claims 56-58 have been added to the application to specific aspects of the invention as fully supported by Applicants' specification. Applicants most respectfully submit that all of the claims now present in the application are in full compliance with 35 USC 112 and are clearly patentable over the references of record.

Applicants have carefully considered the rejection of claims 26, 42, 43, 45, 46, 49, 50, 52 and 53 under 35 U.S.C. 102(e) as being anticipated by Johnston in view of the fact about malted cereal disclosed in Witt et al. (US 4,241,183, of record). This rejection has been carefully considered but is most respectfully traversed.

Applicants wish to direct the Examiner's attention to MPEP § 2131 which states that to anticipate a claim, the reference must teach every element of the claim.

> "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "The identical invention must be shown in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed Cir. 1989). The elements must be arranged as required by the claim, but this is not an *ipsissimis verbis* test, i.e., identity of terminology is not required. *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed.Cir. 1990).
>
> *Akzo N.V. v. International Trade Comm'n*, 808 F.2d 1471, 1 USPQ2d 1241 (Fed. Cir. 1986) (Claims to a process for making aramid fibers using a 98% solution of sulfuric acid were not anticipated by a reference which disclosed using

sulfuric acid solution but which did not disclose using a 98% concentrated sulfuric acid solution.).

It is urged in the Official Action that Johnston teaches a method for preventing diarrhea of young animals by feeding the animals a food product prepared from malted grain, particularly, malted barley. Reference is made to the Abstract. It is then urged that a method of preventing diarrhea is seen to the skilled artisan to possess the process of regulating the flux of fluid and electrolytes in the intestine. The method of Johnston is said to be considered inherently to possess the limitation of "1 ml of blood of said animal will contain at least 0.5 units of antisecretory proteins". This statement in the Official Action is specifically traversed and is believed to be based on an erroneous conclusion as would be appreciated by one of ordinary skill in the art to which the invention pertains. In this regard, Applicants have duly considered the ex parte Novitski et al. decision and submit that the facts of the present case are clearly distinguishable from facts in this decision and the decision is not applicable to the facts of the present case.

It is further noted in the Official Action that Witt et al. teach that it is well known that malted cereal contain enzymes and are widely used in food products. It is therefore concluded that the food product employed by Johnston does have enzymatic activity since it contains malted cereals. Again, this aspect of the rejection is specifically traversed as there is no suggestion of the claim limitation concerning the amount of antisecretory proteins in 1 ml of blood as required by the presently claimed invention.

The present application describes the surprising discovery that malted cereals can be utilized without prior cooking (*ex corpus*) to form, at a sufficient rate, such amounts of sugars and amino acids, so that the desired induction of ASP is achieve. The generation of sugars and amino acids takes place at body temperature and not at the elevated temperatures indicated by Johnston.

The induction or the formation of ASP ultimately requires a certain concentration of sugars and amino acids in the blood. Generally, when food, containing both starch and proteins are eaten, the concentrations of sugars and amino acids, generated by digestion, are too low to induce the rapid formation of ASP, especially when the

individual suffers form the consequences of extreme body fluid flow. By adding the pure substances (sugars and amino acids) in a feed that is fed to an animal, which cannot chose its feed, the necessary concentrations can be achieved. Such a food would not selected by a human with an option of a free choice. It may be noted that free amino acids or enzymes is generally not regarded as a legal food additive. Their use as foods or food enhancers is carefully regulated.

The Examiner claims that the Applicants do not disclose or do not limit the claims to a particular food, which is distinct from prior art. This is not true. In the general description of the invention, it is stated that the use of malt is known in food and feed, but not to the extent that it causes the induction of ASP. Consequently, it is a rational and complete limitation that the cereals and malted cereals are active enough and used to such an extent that the formation of ASP is induced.

Johnston's patent claims contain the use of malted cereals when used in a suspension of water. The present invention does not require the use of water prior to consumption. If the Examiner feels more convenient, the present application's claims could be clarified and amended by adding the word "a sufficient quantity of a dry, particulate foodstuff, comprising cereals having enzymatic activity".

The effect on diarrhea claimed by Johnston is not verified for reasons indicated below. An invention without the claimed effect is not patentable. The examples of Witt do not disclose any effect on the flow of body fluids.

The Examiner claims that the invention is to be anticipated by the invention made by Johnston '225, where the use of malt in calf's feed is claimed not to induce diarrhea by allergic reaction, unlike conventional calf's feeds. However, nowhere in the patent is evidence given to support the claimed inhibitory effect on dehydrating forms of clinical diarrheas. Johnston explains the effect by the removal of allergenic compounds. There is no basis in Johnston alone or as interpreted by Witt of the claimed limitation and it is not inherent in the treatment of diarrhea caused by an allergic reaction as described in Johnston.

It is an obvious difference between curing diarrhea and not causing diarrhea by removing an agent, causing diarrhea. Johnston's patent is directed towards the removal of causes, while the present application is directed towards the extreme body liquid flow into the intestines and other tissues. Such a view represents a novel use of malted cereals and is reflected in claim limitations in the presently claimed invention which are not inherent in the Johnston reference as would be appreciated by one of ordinary skill in the art.

The only proven effect of Johnston's diets is an improved daily weight gain when feeding calves and lamb. Diarrhea is not a clear and definite diagnosis. Diarrhea causes clinical dehydration of varying severity. The clinical state of dehydration can be, and generally is, estimated by determining haematocritic values in blood samples, i.e. the relative volumetric difference between the liquid and cellular phases of blood. Such determinations are made by Johnston (col. 13, lines 21-22; col. 17, lines 17-19). The patent reveals that the "hematocrit values were comparable" in the test and control group of calves. One of ordinary skill in the art reading the patent gets the impression that no significant differences in the hydration status between the groups of animals could be diagnosed. Consequently, it is not proven that the compositions, made according to Johnston's patent, have any significant effect on the inhibitory/modulatory influences on more or less dehydrating forms of clinically verified diarrheas. Any observed, but not reported or documented, difference between the groups, in defecation frequency, stool dry matter content or faecal volume can therefore only be accredited to stochastic incidence.

Obviously, the reported improved weight gain in comparison to the control feed, described in Johnston's patent, could be caused by improved digestibility, accomplished by the pre-treatment of the feed ingredients. Simpler forms of diarrhoea do not reduce the resorption of nutrients The patent does not disclose the duration of the treatment required to accomplish the desired effect.

Johnston's claims describe that soybean meal and (wheat) flour are mixed in a water suspension; that the mixture is gelatinised and that malt is added to this mixture.

The allergenic compounds of the soybean meal are hydrolysed, as the content of starch is hydrolysed, according to known reactions, to glucose and dextrins. The effect of the malted cereal's enzymes on the flour mixture in a dilute water suspension forms an important part of Johnston's invention.

According to the present invention no such pre-treatment of the food is required to achieve the intended induction of antisecretory proteins. The amount of malted cereals required according to Johnston's invention, is much smaller than the amount required by the present invention. The patent description tells that preferably 1-15%, or more preferred 1-10% by dry weight grain malt (col. 3, lines 5-7) is required to improve weight gain in feeding calves and lambs. With such small amount of concentration of malt the intended effect of the present invention will not be achieved. Applicants are mindful of the broad teaching of about 50% malted barley in Johnston, but submit that this is way outside the working examples described in the patent and the preferred range. It is not suggestive or anticipatory of the presently claimed invention as would be appreciated by one of ordinary skill in the art to which the invention pertains.

Products made according to Johnston's invention aim at replacing traditional milk protein-based milk-replacers for weaning calves and lambs, thus the invention aims at solving feeding problems arising when milk proteins are replaced by soybean meal flour. It seems obvious that such suspensions and solutions are not intended for human consumption, nor do they fit an industrial food manufacturing and distribution system and clearly there is no anticipation of claims 49, 50,52 and 53.

Intestinal hypersecretion clinically classified as diarrhoea has been demonstrated to be caused by lack of antisecretory proteins. Hypersecretion in other tissues causes a number of clinical symptoms. Consequently, the inventors summarize the restoration to normal balance between secretion and absorption by indicating a minimum ASP-content in blood. The presently claimed invention specifies that the foodstuff must be composed so that the ASP-content in the blood amounts to at least 0,5 ASP-units. It is not a simple task to compose foods with malted cereals that is eaten voluntarily and

regularly in such an amount that the desired effect is achieved in accordance with the presently claimed invention.

The most common food, made from malted cereals, is beer of various strengths. Repeated trials with different beers have not revealed an increased FIL-content (ASP) in the blood. No reduced secretion of body fluids was observed during these trials. Other known foods, containing malted cereals, cannot be consumed to such an extent that an increased FIL-content in the blood is measurable. It should be remembered that various foods containing, now known as insufficient amounts, malt are known since long. If the effect on diarrhoea caused by malt was obvious and non-novel, it should reasonably have attracted much attention since long. One conclusion is that it is non-obvious and novel that malted cereals can induce the formation of antisecretory proteins.

Consequently, prior art does not disclose that the effects indicated in the present invention was anticipated, neither by Johnston's patent, nor the other cited patents. In reality, the sugar content in malted cereals is low and is controlled by process conditions. The fact that enzymes present in malted cereals can be utilized to saccharify starch is well know, but it is novel that the malted cereals in conjunction with endogenous enzymes can digest food at such a rate and to such an extent that the desired induction of antisecretory proteins is effectively accomplished. This is both non-obvious and novel.

By the induction and formation of antisecretory proteins a general effect against the symptoms, caused by abnormal hypersecretory conditions is achieved. The antisecretory proteins limits all kinds of secretion in tissues, expressed in diseases like inflammatory bowel disease, Crohn's disease, ulcerative colitis, inflammations, arthritis, Meniere's disease, made public through the US patent 6,344,440. The induction and production of antisecretory proteins caused by malted cereals is both novel and non-obvious, considering prior art at the time of the first patent application no. 6, p. 824-829) and a congress report from the XVII World Congress IFOS, Cairo, Egypt 2001, which

are enclosed, are considered. Accordingly, it is most respectfully requested that the anticipation rejection be withdrawn.

The rejection of claims 26, 33, 34 and 41-55 under 35 U.S.C. 103(a) as being unpatentable over Johnston in view of Lange et al. and further in view of Robbins et al., Aspinal et al. and Witt et al. has been carefully considered but is most respectfully traversed for the reasons discussed above.

Applicants again wish to direct the Examiner's attention to the basic requirements of a prima facie case of obviousness as set forth in the MPEP. Section 2143 states that to establish a prima facie case of obviousness, three basic criteria first must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Section 2143.03 states that all claim limitations must be taught or suggested by the prior art. In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). "All words in a claim must be considered in judging the patentability of that claim against the prior art." In re Wilson, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970). If an independent claim is nonobvious under 35 U.S.C. 103, then any claim depending therefrom is nonobvious. In re Fine, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988).

It is the Applicant's firm opinion that Johnston, in combination with Lange et al., Robbins et al. , Wit and Aspinal et al., does not in any way meet the standard which is necessary to establish a prima facie case of obviousness of the presently claimed invention. Thus, as stated above, Johnston teaches a method for preventing diarrhea of young animals, caused by an allergic response to soy flour, and can thus, already for

that reason, not be combined with any of the secondary references to render obvious the presently claimed invention.

It is not obvious for the man skilled in the art that foods, prepared according to the presently claimed invention, have such a powerful effect on patients, diagnosed for ulcerative colitis, Crohn's disease or Meniere's disease as made public from the report discussed above. Nor can such an effect be anticipated by the knowledge from Johnston's patent or the other citations, e.g. that malted cereals can saccharify starch or a more specific knowledge of various starches structures. The present invention cannot be anticipated from information on the kind and amount of amino acids in malted cereals. It should be note that the content of sugars and amino acids is low per se in malted cereals. The works of Robbins and other only show the content of amino acids of the proteins in a sample solution, after sample preparation. Some cited references aim at producing palatable compositions rich in dietary fibre. It is not obvious that utilization of prior art can compose a food product with an antisecretory protein inductive capacity.

The cited references do not indicate that a nutritious industrially manufactured food with a powerful inductive power can be prepared form malted cereals. It can be noticed that products, made according to the invention, are now bought on the market.

On the contrary, the Lange et al patent, U.S. 5,296,243, being the inventors' own publication is acknowledged as prior art in the paragraph bridging pages 1 and 2 of the present application. The comments in therein should be noted. This prior art relates to a process for correcting and optimizing the composition of a feed for regulating the exchange of fluid and electrolytes in the gut of animals by adding certain sugars and amino acids to the feed. The objective is achieved by the fact that the addition to the feed of certain sugars and amino acids induces formation of antisecretory proteins named "feed-induced lectines" (FIL) in this patent. There is no motivation to one of ordinary skill in the art to combine this teaching with that of Johnston et al and arrive at the presently claimed invention.

The object of the present invention is to provide a foodstuff alleviating or remedying the problems and phenomena associated with undesired secretion of body fluids, and this is accomplished, according to the presently claimed invention, by regulating the net flux of fluid and electrolytes in the intestine by the addition of enzymes or compounds providing enzymatic activity. There is no suggestion of this in either Johnston et al or Lange et al, let alone in their combined teaching.

The regulation of the flux of fluid and electrolytes is achieved, according to the present invention, by using products for the preparation of foodstuff having such an enzymatic activity that the foodstuff, when consumed, induces antisecretory proteins (ASP). According to the claimed invention it has been shown that the ASP level required in order to obtain the intended effect is at least 0.5 units of ASP per ml of blood. Any product having enzymatic activity to induce the desired formation of ASP can be used. One of ordinary skill in the art to which the invention pertains, can easily, by routine tests, measure the response to the ASP induction of the foodstuff according to the method stated in U.S. 5,296,243. Briefly, the method involves measuring a standardized secretion response in the small intestine of a rat (cf. also Example 1 of the present application). It is obvious that foodstuffs prepared according to the invention can be varied in a great number of ways and be given by different embodiments. Owing to this, diet monotony can be avoided. The need of stimulation of different individuals to reach an effective ASP concentration can be met by measuring the response of food intake, as stated above. Through the invention, one can also compensate for varying activity of enzyme preparations as well as for differences in enzymatic activity between e.g. malted cereals.

It is to be noted that Lange et al does not disclose or even indicate the control of the amount of formed antisecretory proteins by the use of products having enzymatic activity in the preparation of foodstuff. (As is also evident from the International Preliminary Examination Report, the Examiner of the PCT authority considered all claims new and inventive over the prior art with respect to the corresponding PCT application.)

The use of foodstuff prepared from cereals having enzymatic activity does not require any special permit or acceptance from food inspection authorities. This is one advantage of the invention. Further, the desired effect, induction of antisecretory proteins, is safely and reproducibly achieved. The method for measuring this effect is described in the specification and suitable limits to be achieved are given. The invention enables the user of the invention to produce tasty and varying foods which provide the objective of the invention.

The known use described in Lange et al. (U.S. 5,296,243) of certain sugars and amino acids for achieving indication of antisecretory factor (protein) is in some instances impracticable. Some good inducing amino acids are not allowed as food supplement. The use of sugars and amino acids in prepared (baked, fried etc.) foods can reduce the activity of the prepared food by the formation of so-called Maillard compounds during cooking. The use of sugars can in some instances render the food an excessive unpleasant sweet taste. The invention solves this problem.

Aspinal et al. only show that the amylopectin contained in starch is more affected by the malting of barley than the amylose component that is relatively little degraded. Robbins et al. have determined the amino acid composition of the proteins of the malts of cereal species and has not determined the composition pattern of free amino acids of the malts. The reason why the amino acid composition is changed during malting is that some amino acids of the proteins are metabolized and, owing to that, the total composition of amino acids of the malted cereal proteins is changed. From this reference, the one of ordinary skill in the art cannot conclude how the composition of free amino acids the proteins of the malt is changed during malting and the combined teachings do not render obvious the presently claimed invention.

Consequently, it would not have been *prima facie* obvious to a person of ordinary skill in the art, at the time the claimed invention was made, to employ the method of Johnston for preventing diarrhea of young animals, caused by an allergic response to soy flour, in order to regulate the flux of fluid and electrolytes in the intestine of an animal since Aspinal et al. and Robbins et al. do not at all teach the composition of free

amino acids of the malt upon malting of the cereals. In re Fritch, 23 USPQ 1780, 1784(Fed Cir. 1992) ("It is impermissible to engage in hindsight reconstruction of the claimed invention, using the applicant's structure as a template and selecting elements from references to fill the gaps.). Thus, the Applicants strongly assert that Johnston cannot be combined with Lange et al., Robbins et al. and Aspinal et al. in order to reject claims 21-41 under 35 U.S.C. 103(a). Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 26, 33, 34 and 41-55 under 35 U.S.C. 103(a) as being unpatentable over Bolles et al. and Camburn in view of Witt has been carefully considered but is most respectfully traversed. Again, there must be some motivation in the prior art to modify the references to arrive at the claimed invention. This motivation may not be found in Applicants' specification and "obvious to try" is not the standard of obviousness under 35 USC 103.

Bolles et al. teach the preparation o£ a flaked cereal product having a fiber content, and this is the very purpose of Bolles et al. A starch-degrading enzyme is used to degrade the starch of a bran product in order to prepare an edible and palatable flake product. The process of Bolles et al, is aimed at gelatinizing the starch fraction.

Camburn teaches solubilization of dietary fibers (an alpha- beta-glucan containing foodstuff) comprising processing a carbohydrate-containing material under severe conditions of mechanical disruption and shear at high screw speed in an extruder. Glucans are known to reduce the cholesterol level in blood but not to normalize abnormal fluid secretion in the intestines. Therefore, Camburn does not add any knowledge of how to prepare a foodstuff for induction. of antisecretory proteins in order to achieve such a normalization.

Witt et al. teach the use of the starch-degrading enzymes of the malt to reduce the viscosity of starch pastes by liquefaction. The skilled man cannot, from this reference, draw any conclusions leading to the claimed invention.

Consequently, the combination of Bolles et al., Camburn and Witt et al. to support the rejection is improper since Applicants cannot understand how the claimed

invention, disclosing a method for regulating the flux of fluid and electrolytes in the intestine of an animal by feeding the animal a sufficient quantity of a foodstuff, prepared from cereals having enzymatic activity, so that 1 ml of blood of said animal will contain at least 0.5 units of antisecretory proteins, can be obvious to the skilled man reading Bolles et al., teaching the preparation of a flaked product by using starch degrading enzymes for starch gelatinization, in combination, with Camburn, teaching a mechanical process for solubilizing an alpha-glucan containing foodstuff, and Witt et al., using the starch-degrading enzymes of the malt for liquefaction of starch. The three references all teaches liquefaction or gelatinization of starch in order to obtain edible and palatable cereal products (flakes, dietary fibers) and do not even indicate the use of cereals having enzymatic activity to provide effective amounts of sugars and amino acids to control or govern the formation of antisecretory proteins in the foodstuff so prepared.

Accordingly, it is most respectfully requested that this rejection be withdrawn.

It is further stated in the Official Action that Applicants' remarks with respect to Johnston has been carefully considered. It is noted in the Official Action that a claim is anticipated if any species encompassed by the claim is anticipated. Note the claim method is directed to regulation of the flux of fluid and electrolytes in the intestines which is what Johnston method does. This statement is specifically traversed. As clearly emphasized above, Johnston's method relates to removing the allergic reagent and not, the method in accordance with the presently claimed invention which controls the secretion of antisecretory proteins to a specified amount using an enzyme active cereal.

In further amending the claims, Applicants have more clearly defined the particular foodstuff and the more limited claims are clearly distinguishable over the prior art and are certainly not obvious from these claim limitations.

As clearly stated during the interview on March 7, 2003, with the Examiner, this is a misconception. Malted cereals contain proteins and Aspinal teaches the amino acid composition of these proteins. This means that neither amino acids nor sugars exist *per se* in the malted cereals in a considerable amount. Sugars and amino acids can, of

course, be formed form various plant materials, including malt, by action of acids, bases and enzymes. Malted cereals are known to be rich in enzymes. However, the enzymatic action (for a measurable rate) requires that water be added. Consequently, worth cooking, starch hydrolysis etc. is well known by prior art, as correctly pointed out by the Examiner, includes the addition of water. The hydrolytic action exerted by the malted cereals as described in Johnston's patent is performed in a water suspension. In order to increase the hydrolytic reaction rate the temperature is elevated to some 180°F. Obviously, for anyone skilled in the art, according to the Examiner, when cooking the mixture of starch containing flour and soybean meal with enzymes from malted cereals sugars and amino acids are formed. This fact has not prevented the USPTO, in full view of the prior art, the Lange et al. patent US 5,296,243, application first filed 4 January 1990, to grant Johnston a patent. One can also assume, as Johnston points out, that the allergenic compounds present in soybean meal are hydrolyzed to a certain extent. In the eyes of USPTO Johnston's invention is novel and nonobvious.

In view of the above comments and further amendments to the claims, favorable reconsideration and allowance of all of the claims now present in the application are most respectfully requested.

Respectfully submitted,

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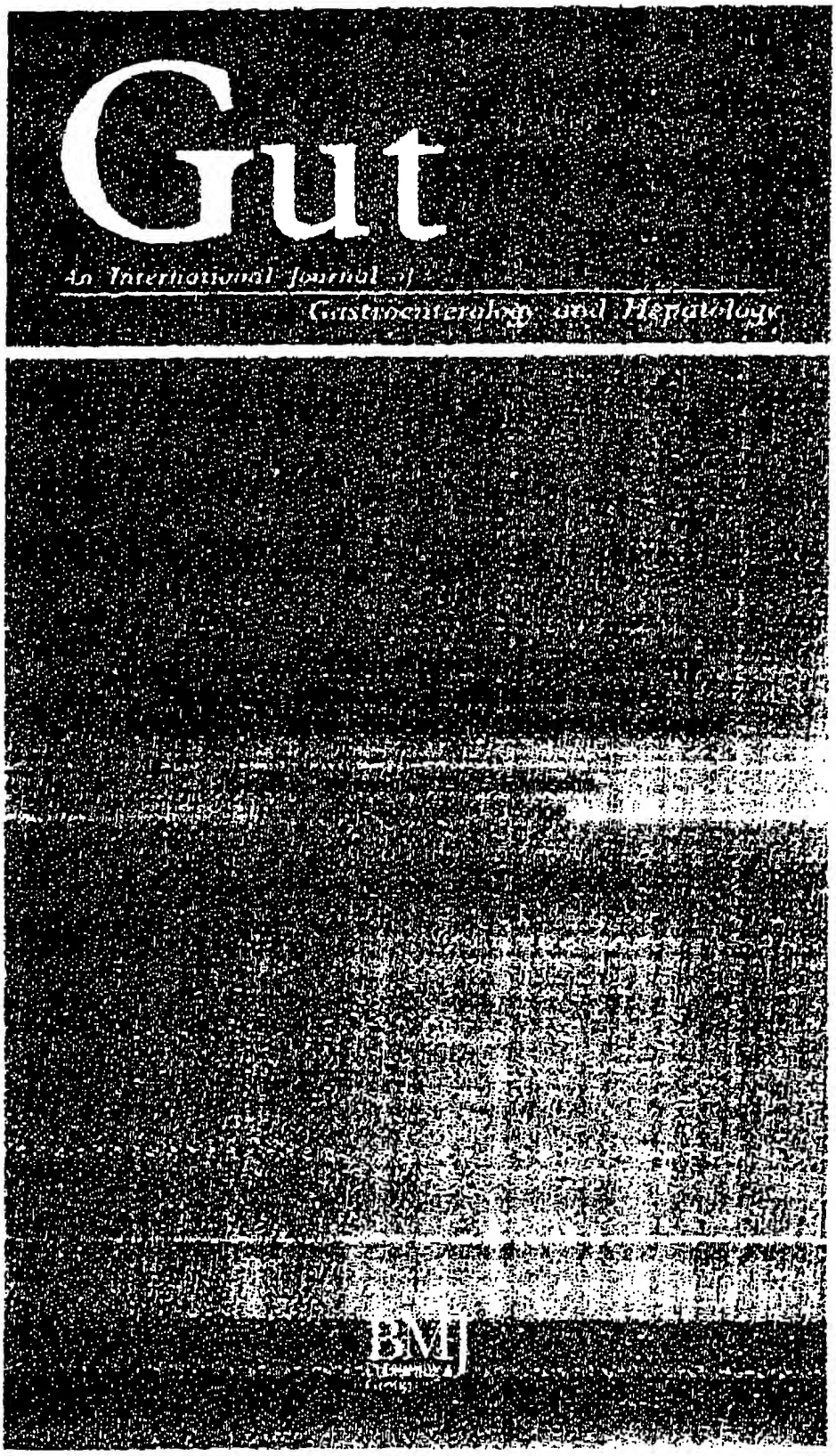
December 2, 2003


Gut
An International Journal of
Gastroenterology and Hepatology
BMJ

Food induced stimulation of the antisecretory factor can improve symptoms in human inflammatory bowel disease: a study of a concept

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Abstract

Background—Antisecretory factor (AF), a 41 kDa cloned and sequenced protein, suppresses intestinal inflammation and hypersecretion in animals. Endogenous AF production can be induced by dietary modifications in several animal species, and this feed has been shown to reduce the incidence of diarrhoeal disease in weaning piglets. The role of AF in intestinal disease in humans is not known.

Aims—To study the effects of hydrothermally processed cereals, optimised for AF induction in animals, added to the diet of patients with longstanding symptoms of inflammatory bowel disease (IBD).

Patients—Fifty three patients with IBD (ulcerative colitis and Crohn's disease) were entered into the study, and 50 completed follow up. The experimental group consisted of 16 females (mean age 50 (SEM 5) years) and 10 males (41 (4) years) and the placebo group of 12 women (41 (4) years old) and 12 men (51 (5) years).

Methods—Patients were randomised to receive either hydrothermally processed cereals (active treatment) or the same amount of ordinary cereals (placebo treatment) for four weeks in a double blind study design. Baseline diet and medications remained unchanged. Bowel symptoms, plasma levels of AF, and colonic biopsies were evaluated before and after treatment.

Results—The active treatment significantly improved subjective ratings of clinical symptoms and increased plasma AF levels compared with placebo. Plasma lipid levels were unaffected.

Conclusion—Hydrothermally processed cereals can induce AF production in human IBD. This increase in endogenous AF activity is associated with clinical improvement. Further studies are warranted to clarify the exact role of AF in human intestinal disease.

(*Gut* 2000;46:824–829)

Keywords: antisecretory factor; functional food; ulcerative colitis; Crohn's disease

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Accepted for publication 25 November 1999

We have isolated, cloned, and sequenced "antisecretory factor" (AF), a 41 kDa protein.[1] AF exerts an inhibitory effect on various experimental diarrhoea models in the rat and pig small intestinal ligated loops. Thus only 10^{-11} M to 10^{-12} M of recombinant AF is required to revert intestinal hypersecretion in vivo when added from both the mucosal and the serosal side of the epithelium. Recombinant AF also inhibits the inflammatory response induced by toxin A from *Clostridium difficile* in rat jejunal loops.[2]

Over the past decade we have been working in close cooperation with European farmers on improvement in feed quality for meat producing animals. It has been proved that optimally designed feed reduces diarrhoea and increases daily weight gain in newly weaned pigs better than that achieved by traditional high protein feed supplemented with antibiotics.[3] AF has proved to be one of the most useful laboratory variables in the design of optimal feed composition for livestock.[4]

In the present study we evaluated food optimised for AF induction in humans suffering from intestinal disease. Thus patients with a clinical condition associated with pathological intestinal secretion and/or intestinal inflammation were selected. A double blind study was designed in patients with inflammatory bowel disease (IBD) (ulcerative colitis or Crohn's disease). Two criteria had to be fulfilled: (1) patients should have a longstanding history (more than two years) of intestinal dysfunction and a diagnosis of ulcerative colitis or Crohn's disease, but should be in a stable clinical condition making outpatient treatment possible and; (2) conventional medical treatment and the patient's normal food habits should not be changed.

Patients were randomised to one of two groups: group A, experimental group, received hydrothermally processed cereals capable of increasing plasma AF levels (active food); group B, control group, received the same type of food but the hydrothermally processed cereals were replaced by ordinary cereals without specific AF inducing properties (placebo food). Active and placebo food had the same taste, appearance, and content of energy and nutrients.

The biological role of AF in human intestinal diseases is largely unknown. Thus we hypothesised that the active food would induce AF and affect the clinical outcome of intestinal disease in humans. Consequently, the investigation was designed to study a concept (that is, to evaluate if an increase in endogenous AF in

Abbreviations used in this paper: AF, antisecretory factor; VAS, visual analogue scale; IBD, inflammatory bowel disease; CRP, C reactive protein.

patients suffering from IBD could affect the clinical outcome of the disease).

Material and methods

PATIENTS AND METHODS

The study design was approved by the human ethics committee of Göteborg University. Patients subjected to the active treatment (experimental group) received a diet supplemented with hydrothermally processed cereals capable of endogenous AF stimulation. The control group was given the same type of food but the hydrothermally processed cereals were replaced by ordinary cereals (placebo group). A control panel, responsible for food production, ensured that no difference in taste or quality could be detected between the active and control foods. A delivery firm ensured that the correctly coded cereal product was delivered at the home of the participants. The code of the study was kept at the Swedish "Notarius Publicus" and was not broken until all of the clinical and laboratory results had been registered.

Patients attending an outpatient gastroenterological clinic (head of clinic, SB) with a diagnosis of ulcerative colitis or Crohn's ileitis or colitis were invited to participate in the study. All participating patients had longstanding (>2 years) gastrointestinal disorders resulting in loose stools or frank diarrhoea, and an abnormal number of bowel movements each day, despite conventional medical treatment. All patients had previously undergone biopsies of the rectal mucosa, and the histology of these biopsies verified the clinical diagnosis of ulcerative colitis or Crohn's disease. Thus the selection of patients fulfilled the criteria of Truelove and Witts [5]

Currently, approximately 500 patients with IBD attend the clinic. All 53 eligible patients agreed to take part in the study: 50 patients had chronic ulcerative colitis (29 total colitis, 21 distal colitis) and three patients had Crohn's disease (one ileal disease, two colonic disease). Patients were randomly assigned a daily intake of products containing a certain amount of either hydrothermally treated cereals (active food, capable of endogenous AF stimulation, experimental group) or ordinary cereals (placebo food, without measurable effects on endogenous AF synthesis). Patients were instructed to have a daily intake of 1 dl of muesli, 100 g of pasta cooked for 4–5 minutes, and a roll. They were also informed that one roll was equal to 1 dl of muesli or 50 g of pasta.

All patients reported that they replaced a major part of their ordinary cereal intake with the cereal food used in the study. However, neither the overall diet nor the time schedule for daily food consumption was affected by cereal intake during the test period.

The cereals of the active food were treated hydrothermally in a process similar to malting. The hydrothermal process was continued until the content of sugars and amino acids of the products were in the range given in table 1. After processing, the cereals were dried to 10% moisture content. Sugar content was assayed by soaking 5 g of cereal products for 10 minutes at room temperature in 100 ml of a buffer consisting of 20 mM Na_2HPO_4, 6 mM NaCl, pH 7. Samples were taken from the solution and analysed for sugars and amino acids by HPLC and standard procedures.

Table 1 Content of sugars and amino acids in the cereals of the active food before and after the hydrothermal process. The hydrothermal process was continued until the content of sugars and amino acids of the products were in the ranges given

Content	Before the process (mg/g DM)	After the process (mg/g DM)
Glucose	0.3–0.4	0.6–5.3
Fructose	0.3–0.4	0.6–3.1
Sucrose	8.4–16.4	16.8–65.7
Maltose	0.0	4.0–9.0
Histidine	0.0	0.06–0.25
Glutamic acid	0.12–0.20	0.42–0.44
Lysine	0.03–0.06	0.15–0.29
Tryptophan	0.09–0.22	0.28–0.45
Isoleucine	0.0	0.05–0.30

Both active and placebo food had the same energy and nutrient content. The food consisted of muesli, rolls, and frozen fresh pasta and was produced by BioDoc AB (Box 30 192, S-104 25 Stockholm, Sweden).

PATIENT REGISTRATION

All patients kept a diary over a six week period noting the number of bowel movements per day and the consistency of the stools (hard, solid, loose, or watery). At the end of the study each patient and the principal investigator (SB) evaluated the effect of the added cereal diet on the patient's bowel habits. Clinical improvement or clinical deterioration was plotted as a percentage on a visual analogue scale (VAS score) and expressed as percentage change. In principle, this implies that a score of 100% or more represents total normalisation of intestinal function whereas a score on the minus part of the scale is correlated with subjective deterioration of a multitude of clinical functions related to the intestine. There was good correlation between patient registrations in the diaries and VAS scores.

CLINICAL EXAMINATION

All patients were thoroughly investigated by endoscopy, biopsies, radiography, blood tests, stool cultures, and malabsorption tests at least six months before the start of the study. Most patients had suffered from their disease for several years (minimum two years, maximum 25 years). Endoscopy with biopsies was performed in each patient at the beginning of the study and at the end of the four week period of the active or placebo diet. All biopsies were obtained in a standardised way, 10 cm proximal to the linea dentata in the posterior wall of the rectum.

Plasma was collected from each patient just before start of the study and at weeks 4 and 8 of the study period. Plasma samples were stored at −20°C until tested. Some of the patients underwent left sided colonoscopy at the end of the study.

HISTOLOGY

All rectal biopsies were taken after obtaining informed consent from patients, and were

carefully selected via the endoscope. The biopsies were immersed in 4% paraformaldehyde in phosphate buffered saline (PBS 145 mM, NaCl buffered to pH 7.2 with 70 mM Na_2PO_4), frozen in liquid nitrogen, and 7 µm thick cryostat sections were prepared. The sections were first incubated with the affinity purified antiserum against the C terminal of the AF protein[1] diluted 1/600 and then with alkaline phosphatase conjugated swine antirabbit immunoglobulins (Boehringer Mannheim). The immune reaction was visualised by incubating the sections in a substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate tolodium salt (Boehringer Mannheim). Serial sections were stained with periodic acid-Schiff stain (PAS) and haematoxylin.

To classify AF positive cells, staining of serial sections from the biopsies with various T cell antisera (CD3, CD4, CD8, CD20, CD45RO, and CD68, results not shown) was performed. Thus the position in the tissue in addition to the gross morphology of the AF positive cells were compared with the same markers for the cells appearing positive with the various T cell antisera. The results indicated that the AF positive cells were either CD4 or CD8 positive, and that AF cells could tentatively be classified as a subgroup of T cells.

PREPARATION OF AF AND TEST OF AF POTENCY

AF was purified from plasma using affinity chromatography as previously described.[1] In brief, after passage of the plasma through a small agarose column (Sepharose 6B, Pharmacia LKB Biotechnology, Stockholm, Sweden), the agarose adsorbed AF was eluted with 1 M methyl-α-D-glucoside. The eluate was dialysed against PBS for 24 hours at 4°C and then stored at −20°C until use.

The potency of the purified AF was determined by the ligated loop assay in rats using cholera toxin as a secretagogue.[8] In brief, under ether anaesthesia, one loop, about 10 cm in length, was made in the jejunum. The AF test substance (2 ml), prepared from the plasma of the patients as described above, was administrated intravenously via the dorsal vein of the penis. The loop was then challenged with 3 µg of cholera toxin diluted in 1.5 ml of PBS (0.15 M NaCl, 0.05 M Na_2HPO_4, pH 7.5). The abdominal wall was then closed and the animal was allowed to wake up. The duration of the challenge was five hours after which the rat was killed by cervical spine dislocation, the abdomen opened, and the loop dissected out. Net fluid secretion (mg/cm) was estimated by subtracting the weight of a control loop from that of the experimental animal loop. The AF preparation with 50% inhibition of fluid secretion was assigned an AF value of 1.0.

STATISTICAL ANALYSES

Data are presented as mean (95% confidence intervals) unless otherwise stated. Variables were first examined for normal distribution using the Komolgorov-Smirnov test. Group means for non-normally distributed variables were compared using the Mann-Whitney test. Group means of variables without significant deviation from normality were compared using the Student's t test. Differences in proportions were assessed by χ^2 tests (Pearson χ^2 and Cochran's linear trend). Correlations were examined using Spearman's rho. A p value of 0.05 or less was considered significant. All calculations were made using SPSS version 7.5 software (SPSS Inc., Chicago, Illinois, USA).

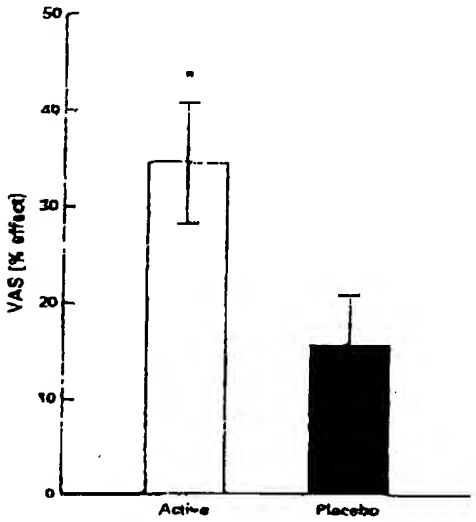

*Figure 1 Subjective assessment of the effects of the two diets after four weeks of treatment, expressed as percentage improvement of the condition (VAS). Data are mean (SEM). *Significant difference between the active and placebo groups ($p<0.05$). VAS, visual analogue scale.*

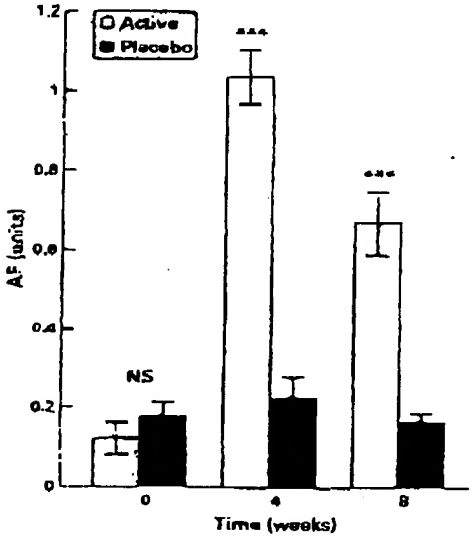

*Figure 2 Plasma levels of antisecretory factor (AF) in the two groups of patients at the start of the experiment, after four weeks of active or placebo diet, and after eight weeks (that is, four weeks after termination of the diets). Data are mean (SEM). ***Significant difference between groups ($p<0.001$) at four and eight weeks. NS, not significant.*

Table 2 Histopathological rating of biopsies

I	Few AF positive cells, no acute inflammatory reaction
II	Moderate number of AF positive cells, no acute inflammatory reaction
III	Large number of AF positive cells, no acute inflammatory reaction
IV	Moderate number of AF positive cells, acute inflammatory reaction with ongoing crypt destruction
V	Moderate number of AF positive cells, acute inflammatory reaction with extensive crypt destruction

Results

PATIENT RESULTS

Fifty of 53 patients completed the whole study: two patients in the placebo group withdrew from the study after 11 and 15 days, respectively, because of abdominal discomfort; one patient in the active group was excluded because of severe recurrence of his illness during the study. A detailed retrospective investigation of these three patients (anamnestic and laboratory values) provided no further information on why these patients terminated the study. A correlation between intake of placebo or active cereals and intestinal/clinical discomfort seems highly unlikely.

The distribution of the remaining patients was as follows, grouped according to the randomised double blind coding: of 26 patients given hydrothermally processed cereals, 16 were women (total ulcerative colitis n=13, distal ulcerative colitis n=2, Crohn's disease n=1; mean age 49.6 (SEM 5.1) years) and 10 were men (total ulcerative colitis n=6, distal ulcerative colitis n=3, Crohn's disease n=1; mean age 41.2 (5.8) years). Of the 24 patients in the placebo group, 12 were women (total ulcerative colitis n=5, distal ulcerative colitis n=7; mean age 40.9 (3.7) years) and 12 were men (total ulcerative colitis n=5, distal ulcerative colitis n=6, Crohn's disease n=1; mean age 51.1 (4.5) years).

The clinical effects of the active and placebo diets were evaluated using the diaries kept by the patients during the two weeks before the study and for the four week study period. The hydrothermally processed cereals caused more solid stools and decreased the frequency of bowel movements compared with the placebo diet. The subjective estimates by the patients on the visual analogue scale (VAS), expressed as percentage change in subjective condition (fig 1), showed a significant difference between the active and placebo diets (active mean +34.6%, n=25, one patient in the active group did not complete this evaluation; placebo mean +15.4%, n=24, difference 19.2, confidence interval 1.8–36.5; $p<0.05$). Chi-square tests were also performed on this material. Patients' estimations of the treatment effects were classified into three groups: (1) worse, (2) unchanged, or (3) improved, according to their VAS ratings. None in the active and one patient in the placebo group reported worsened symptoms, six in the active and 15 in the placebo group reported no change, while improvement was reported in 19 patients in the active group and in eight in the placebo group (Pearson χ^2 9.32, df=2, p=0.009; Cochran's linear trend 9.27, df=1, p=0.002). Conclusively, irrespective of the statistical treatment of the patient material, the difference in VAS ratings between the active and placebo groups remained significant.

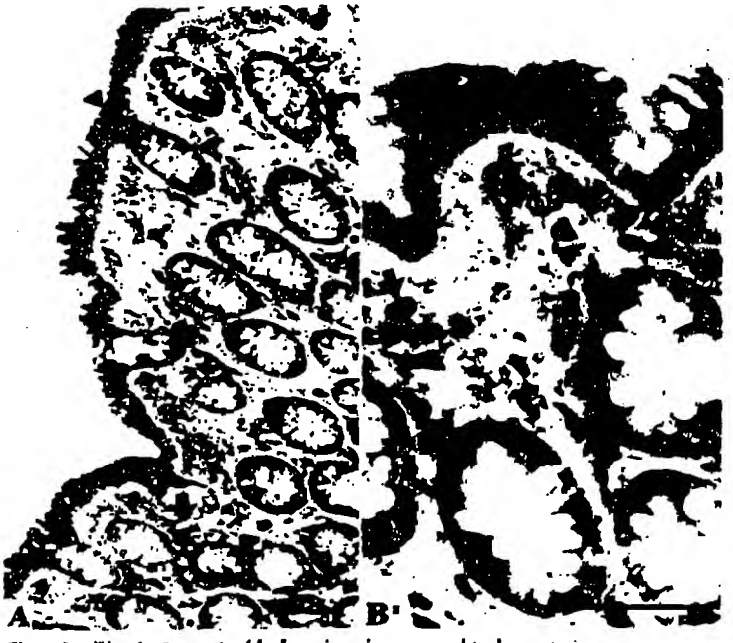

Figure 3 Biopsies from a healthy large intestine processed to demonstrate immunoreactivity of antisecretory factor (AF). Bars=100 μm. (A) Low magnification showing AF immunoreactivity in the surface epithelium (arrowhead), in crypt cells, and in lymphocyte-like cells (arrows) in the lamina propria. (B) Larger magnification showing the AF positive lymphocyte-like cells (arrows).

ANALYSIS OF AF LEVELS IN PLASMA

The results of the AF unit test (fig 2) demonstrated no difference between the active and placebo groups at the start of the experiment (day 0). At the end of the test period (four weeks), AF levels in the active group were greatly increased, resulting in a significant difference between the active and placebo groups ($p<0.001$). AF values declined slowly after termination of active food intake. However, a significant ($p<0.001$) difference between the active and placebo groups still persisted four weeks after termination of the diet (that is, eight weeks after the start of the test).

At the start of the study, elevated levels of C reactive protein (CRP) (>10 g/l) were observed in eight patients in the active group and in two in the placebo group, resulting in a significant difference ($p<0.05$) between groups. There was no difference between groups in samples obtained four and eight weeks after the start of the study. There were no differences between groups in blood lipid values (total cholesterol, HDL cholesterol, and triglycerides), which were within normal limits in all patients (data not shown).

Figure 4 Rectal biopsies from patients with ulcerative colitis treated with the active diet. The sections were incubated with an antiserum against antipracsuory factor (AF). Bars=100 μm. (A) Patient No 31 before the diet period. (B) Patient No 31 after the diet period. There appears to be more AF positive cells (arrows) in the lamina propria after the diet period. (C) Larger magnification of (B), showing AF positive cells (arrows) in the lamina propria. (D) Patient No 12 before the diet period. There are signs of an acute inflammatory reaction. Many small inflammatory cells show moderate AF immunoreactivity. (E) The same patient after the diet period. In this biopsy there is no acute inflammation. Large AF positive cells (arrows) are evenly distributed within the entire lamina propria.

Table 3 Number of biopsies (n) with various histopathological ratings from patients with inflammatory bowel disease, before (A) and after (B) the diet period

Rating of biopsy	Active diet		Placebo diet	
	A (n)	B (n)	A (n)	B (n)
I	9	7	9	9
II	7	11	8	8
III	4	6	5	5
IV	2	0	3	2
V	2	0	3	4

Table 4 Effect of treatment on extent of acute inflammation. Comparison of paired A and B rectal biopsies

Effect on acute inflammation	Active diet (n)	Placebo diet (n)
Worse	0	5
No change	20	13
Improved	4	5

HISTOPATHOLOGICAL EXAMINATION OF RECTAL BIOPSIES

In each patient one biopsy was taken before (A) and one after (B) termination of the diet period. The biopsies were evaluated blindly and the morphological appearance of the two biopsies were compared according to the five groups of criteria listed in table 2. The volume of material in some biopsies in both groups of patients was too small to prepare reliable sections. Such material was excluded from the study. In 47 patients both biopsies were evaluated: of these 24 were from the active group and 23 from the placebo group.

In our ongoing research in healthy volunteers (unpublished), AF immunoreactivity in specimens from such subjects was found in the epithelial lining of the intestine and in mononuclear cells in the lamina propria (that is, similar to the results of cellular localisation demonstrated in fig 3). The most distinct staining was seen in relatively large mononuclear cells with a general appearance of lymphocytes. Staining of serial sections with various T cell antisera (CD3, CD4, CD8, CD20, CD45RO, and CD68; results not shown) indicated that the AF positive cells consisted of a subgroup of T cells and could be either CD4 or CD8 positive. However, a more definite classification of the AF positive cells remains to be done for both gross morphology and cell category.

In patients with acute inflammatory reactions, weak AF staining was also seen in many smaller inflammatory cells. Evaluation of the biopsies was based on the relative number of AF positive cells in the lamina propria and on the presence or absence of active inflammation with ongoing destruction of the crypts (fig 4).

Table 3 shows the morphological ratings of the patients before (A) and after (B) the diet period. To evaluate the effect of the treatment on acute inflammation in the individual case, the histology of A and B biopsies was compared and the extent of acute inflammation after the diet period classified as worse, unchanged, or improved (table 4). Analysis of the paired data in table 4 using the Pearson χ^2 test revealed a significant difference between the active and placebo diets concerning the sections demonstrating acute inflammation after the diet period (χ^2 6.578, df=2, p=0.037), while Cochran's linear trend did not reach statistical significance (1.123, df=1, p=0.29). There were no significant differences in the relative number of AF positive cells between the A and B biopsies in any group. Furthermore, there was no correlation between AF levels in blood samples and the relative number of AF positive cells in the biopsies in any group.

Discussion

Our study showed that AF can be induced in humans suffering from IBD using hydrothermally processed cereals, and that this increase in AF in blood is associated with a reduction in clinical symptoms. Thus a four week period of optimised, hydrothermally processed cereal diet led to subjective and objective improvements in the clinical course of IBD. In accordance with

previous experimental studies in rats[7 8] and pigs[9] as well as field studies in pigs,[3 4] it was found that a specific composition of the diet was followed by increased AF activity in plasma samples. Furthermore, an increase in AF activity correlated positively with patients' own subjective assessments of clinical improvement. Our limited study did not reveal any difference in the response to the diet between patients suffering from total or distal ulcerative colitis. A significant increase in the patient material is required to answer this clinically important question. The two patients with Crohn's disease who received the active diet both responded with significant improvement (VAS +100% and +50%, respectively). Irrespective of the statistical methods used for analysis of the patient material, the difference between treatments remained significant when evaluated by symptom.

Randomisation gave a homogeneous distribution with regard to sex and age in the active and placebo groups. Except for one initial high CRP value in the active group, no difference in CRP values between the two groups was found during the course of the study. No effect on blood lipids was observed with either diet, indicating that the hydrothermally processed cereals did not affect lipid metabolism. However, these results should be evaluated with care as blood samples were not obtained under fasting conditions.

In accordance with previous studies in pigs[10] the AF antiserum recognised surface and crypt epithelial cells and mononuclear cells in lamina propria in the biopsies from patients. These cells have the general appearance of large lymphocytes and are probably made up of a subgroup of T lymphocytes. Participation of T lymphocytes in Crohn's disease has been well documented[11] and so also the case for these cells in ulcerative colitis.[12] However, we have been unable to find a single CD marker that conclusively identifies the entire AF positive population. The morphological similarities between AF positive cells and lymphoid cells is of major interest as various forms of immune phenomena are believed to play an important role in the pathogenesis of tissue damage both in Crohn's disease and in ulcerative colitis.[13 14] Furthermore, the use of immunohistochemistry in combination with in situ hybridisation in IBD may provide important information on the various factors involved in the genetics,[15] aetiology, or pathogenesis of these diseases.[16–18]

In biopsies from patients receiving the active diet there was a tendency to a larger number of AF positive cells in the lamina propria and to a decrease in crypt destruction compared with biopsies from patients receiving the placebo diet. However, when considering only no acute inflammatory reaction versus acute inflammatory reaction, a difference was seen between the active and placebo diets, suggesting a positive effect of the diet on inflammatory status. These data, however, should be interpreted with caution as the total number of patients in the study was comparatively small and the incidence of acute inflammatory changes in the biopsies was low in the total material. This was also reflected in the statistical analyses as the Pearson χ^2 test revealed a significant difference between groups while the linear trend test failed to reach statistical significance.

We did not find any correlation between AF activity in blood samples and staining of AF positive cells in the rectal biopsies analysed by immunohistochemistry. Previous northern blot and in situ hybridisation analyses indicated that AF mRNA is expressed in several peripheral tissues in addition to the initially described expression in the pituitary gland.[10] We do not know the source of the dietary induced AF in blood but it is unlikely that serum AF reflects AF produced in the rectum. The increase in serum AF in response to the active diet might tentatively be due to AF synthesis in other parts of the intestinal tract, for example the small intestine. Further studies are needed to clarify this question.

In conclusion, this study demonstrates the possibility of inducing AF in IBD patients by supplementing their conventional diet with hydrothermally processed cereals. The increase in AF in plasma samples was associated with clinical improvement. Further studies are warranted to elucidate the role of AF in human intestinal disease.

Financial support was provided by the AS Factor AB, Nocon AB, the Swedish State under the LUA agreement (grant No I 33 913), and the Swedish Medical Research Council (grant No 12 611).

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Abstract

TITLE

INDUCTION OF ANTISECRETORY FACTOR IN MENIÈRE'S DISEASE.

AUTHOR(S)

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IDEA

Antisecretory factor (AF) is a protein affecting intestinal inflammation and secretion. AF is proposed also to have effect on the production of endolympha in the inner ear. Endogenous AF production can be induced by peroral intake of amino acids and monosaccharides found in specially processed cereals.

METHOD

Thirty patients with severe Menière's disease were studied. All were treated with cereals which induce AF. Audio-vestibular tests were performed and the levels of ASF in blood were determined before, during and after the treatment. Immunohistological investigations were performed to study the AF location in tissues.

RESULTS

Increased levels of AF in blood were demonstrated in 80% of the patients and were correlated to beneficial outcome of the clinical symtoms. Immunohistologically AF was demonstrated in cerebellum, and in the inner ear in experimental animals.

CONCLUSION

Induction of AF by treatment with cereals seems to affect the endolymphatic production and has a positive effect on the symtomatology in Menieres disease. AF location both to the inner ear and CNS may indicate a new regulatory function of the auditive and vestibular functions.

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